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Evaluation of the BeTha Gene 1 Kit for the Qualitative Detection of the Eight Most Common Mediterranean β-Thalassemia Mutations

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We describe the evaluation of the Bio-Rad BeTha Gene 1 kit (Bio-Rad Laboratories, Hercules, CA), a DNA-probe assay designed for the qualitative determination of the eight most common Mediteranean β-thalassemia mutations. The kit utilizes the principle of allele-specific oligonucleotide (ASO) hybridization. Following sample preparation and in vitro DNA amplification by the polymerase chain reaction (PCR), an allele-specific detection of the amplified products by a nonradioactive enzymatic assay is performed. Genomic DNA is prepared from an individual's whole blood with a DNA purification matrix. In a second step, the β-globin gene is amplified in a multiplex PCR reaction containing four 5′ biotinylated oligonucleotide primers. In a final step, an aliquot of the PCR reaction is first chemically denatured and then captured in two eight-well strips of a 96-well enzyme-linked immunosorbent assay (ELISA) plate by hybridization to an immobilized ASO probe. Each DNA sequence at each of the eight mutation sites is represented by one normal and one mutant ASO. During this capture/hybridization step, which is performed at 37°C, only perfectly matched PCR products will be captured by an ASO. Subsequently, the allele-specific captured biotin-labeled PCR products are detected by a colorimetric enzymatic reaction. The system permits the detection of 16 β-thalassemia alleles using a high-throughput format that can be automated easily. A clinical feasibility study was performed to evaluate the functionality (method camparison study, assay validity using samples previously collected and stored at various temperatures for different periods of time, interference on kit performance, and assay validity for prenatal diagnosis) and the usability (ease of use, sample throughput) of the kit. The analysis of 110 samples previously studied with reference methods showed 100% clinical sensitivity and specificity. We demonstrate here that the procedure not only increases the throughput of β-thalassemia allele genotyping but also provides an accurate, rapid, reliable, and nonisotopic diagnostic tool. Am. J. Hematol. 59:214–222, 1998.

Key words: β-thalassemia; Mediterranean; polymerase chain reaction; allele-specific oligonucleotide (ASO) hybridization; colorimetric detection

INTRODUCTION

β-Thalassemia is a group of autosomal recessive diseases all characterized by a reduced synthesis of the β-globin chain [1]. As a consequence of the decreased synthesis of the β-globin chain, α-globin is in excess and forms tetramers that are insoluble and precipitate within red blood cells, leading to their premature destruction in the bone marrow and marked trapping in the spleen. Red cells from individuals with β-thalassemia are reduced in size, and their hemoglobin (Hb) A2 levels are increased.

In the past 17 years, the molecular bases of β-

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thalassemia have been largely elucidated. In fact, more than 180 different molecular defects in the β-globin gene that produce these disorders have been characterized [2]. However, different geographical regions have different groups of mutations, consisting of a few common ones along with varying numbers of rare ones. In the Mediterranean area, the frequency and distribution of the β-thalassemia mutations are not homogeneous [3]. However, eight mutations (CD39, IVS1:110, IVS1:6, IVS1:1, IVS2:745, IVS2:1, −87, and CD6-A) account for more than 90% of the β-thalassemias in the region.

Although most heterozygous individuals for β-thalassemia are clinically asymptomatic with minor hematological abnormalities, they represent a potential risk since they can transmit the β-thalassemia gene to their offspring. Homozygous individuals for β-thalassemia usually have β-thalassemia major, which is characterized by dependence on blood transfusions to sustain life. Although bone marrow transplantation has been used with success in a limited number of patients, there is no definitive cure except supportive management. Therefore, for the present, prevention of the birth of affected children by heterozygous screening, genetic counseling, and prenatal diagnosis have proven to be the most effective management for these disorders [4].

The use of molecular techniques has been widely documented to confirm the diagnosis of β-thalassemia and, more importantly, for prenatal diagnosis [5]. Polymerase chain reaction (PCR) [6] has been coupled with a great variety of approaches such as restriction endonuclease analysis [7], dot-blot analysis with ASO probes [8], allele-specific PCR [9], reverse dot-blot [10], competitive oligopriming [11], and DNA sequencing [12], to determine the presence of known β-thalassemia mutations on a DNA template. Although rapid and reliable, these techniques are not convenient for use in clinical laboratories because they are not easily amenable to automation. Here we report a study conducted to evaluate the performance characteristics of the BeTha Gene 1 kit, a DNA probe test to diagnose β-thalassemia. The procedure was found to be sensitive, specific, reproducible, and rapid.

MATERIALS AND METHODS

Specimens

Three laboratories trained to perform diagnosis of β-thalassemia by DNA analysis participated in this evaluation and were designated as A (Servizio di Prevenzione e Terapia della Thalassemia, Palermo), B (Istituto di Clinica e Biologia dell'Eta Evolutiva, Cagliari), and C (Thalassemia Center- Laikon Hospital, Athens), respectively. All reagents and equipment for this study were supplied by Bio-Rad Laboratories (Hercules, CA). The method comparison study was performed on whole blood samples collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes from 110 individuals with known β-thalassemia genotype (see genotype distribution in Table I). In 77 cases, genomic DNA was prepared using standard procedures [13] and stored at −20°C until analysis. For the remaining 33 samples, genomic DNA was prepared from blood immediately after collection using the Instagene™ whole blood kit (Bio-Rad Laboratories). The Instagene matrix included in the kit is made with a specially formulated chelex resin [14]. This DNA preparation procedure provides PCR-ready DNA in 30–45 min.

TABLE I. Genotypes and Number of Samples Studied for Method Comparison Study

Genotype	Number of samples studied
CD39/CD39	8
CD39/βA	7
CD39/IVS1:110	2
CD39/IVS1:6	2
CD39/IVS1:1	1
CD39/IVS2:745	1
CD39/IVS2:1	3
CD39/−87	3
CD39/CD6-A	3
IVS1:110/IVS1:110	8
IVS1:110/βA	9
IVS1:110/IVS1:6	2
IVS1:110/IVS1:1	1
IVS1:110/IVS2:745	1
IVS1:110/−87	1
IVS1:110/CD6-A	1
IVS1:6/IVS1:6	7
IVS1:6/βA	10
IVS1:6/IVS1:1	2
IVS1:6/IVS2:745	1
IVS1:6/IVS2:1	1
IVS1:6/−87	3
IVS1:1/IVS1:1	1
IVS1:1/βA	4
IVS1:1/IVS2:745	2
IVS1:1/−87	2
IVS2:745/IVS2:745	1
IVS2:745/βA	4
IVS2:745/−87	2
IVS2:1/IVS2:1	2
IVS2:1/βA	3
−87/βA	3
CD6-A/CD6-A	1
CD6-A/βA	3
βA/βA	6
Total genotypes = 35	Total samples = 110

For the interfering genotypes study, genomic DNA was prepared from EDTA anticoagulated whole blood samples using standard protocols and stored at −20°C until analysis. For the storage study, two EDTA anticoagulated whole blood samples were collected from normal (AA) individuals and genomic DNA was extracted from these samples using the Instagene whole blood kit.

Fetal DNA was prepared from chorionic villi using the procedure described by Kazazian [15].

In Vitro Amplification of the β-Globin Gene and Genotype Detection Assay

Ten microliters of Instagene-extracted genomic DNA or 10 μl of 25 ng/μl DNA (if extracted with other procedures) were pipetted into 200 μl reaction tubes. This was followed by the addition of 40 μl of reaction mix (Amplification Master Mix plus two U of AmpliTaq DNA Polymerase, Perkin-Elmer, Foster City, CA). The Amplification Master Mix reagent consists of PCR buffer, four biotinylated primers (β-Th1 = 5′ TAAGCCAGTGCCAGAAGAGCC, Ph1 = 5′ CAATCATTCGTCTGTTTCCCATTC, βTh2 = 5′ CACTGACCTCCCACATTCCC, Ph2 = 5′ AATGTATCATGCCTCTTTGCACC), and deoxynucleotide triphosphates dATP, dCTP, dGTP, and TTP. The assembly of the PCR reactions was done in a separate room devoted only to sample preparation and PCR reactions set-up. Subsequently, the tubes were transported into a different room and placed into a thermal cycler programmed for one cycle of one min at 94°C; 34 cycles of 45 sec at 94°C, 15 sec at 62°C, and 30 sec at 72°C; and one cycle of 45 sec at 94°C, 15 sec at 62°C, and five min at 72°C.

After the amplification of the β-globin gene, 85 μl of denaturation solution reagent, 68 μl of H_2O, and 17 μl of each amplified DNA sample were mixed in a tube and incubated at room temperature for 10 min. For each DNA sample, a 40 μl aliquot of the hybridization buffer reagent was added into each well on one green-colored strip containing immobilized allele-specific oligonucleotide (ASO) for the normal alleles, and one white-colored strip containing immobilized ASO for the mutant alleles. Subsequently, 10 μl of denatured PCR products were transferred into each well. After a 60-min incubation at 37°C to allow hybridization, the strips were washed five times with 1× well wash buffer in a microplate washer. A 50 μl aliquot of streptavidin-horseradish peroxidase (SA-HRP) conjugate reagent was then added into each well. After the incubation with the enzyme conjugate for 30 min at 37°C, the wells were again washed with 1× well wash buffer five times. Subsequently, a 50 μl aliquot of 3,3′-5,5′ Tetramethylbenzidine (TMB) reagent was added to each well and incubated at room temperature for 10 min. Following incubation, each well received 50 μl of stop solution reagent to stop color development, and the difference in the absorbance at 450 nm vs. 655 nm of each well was determined (A450–655).

RESULTS

The eight most common Mediterranean β-thalassemia mutations, CD39, IVS1:110, IVS1:6, IVS1:1, IVS2:745, IVS2:1, −87, and CD6-A were selected to assemble the

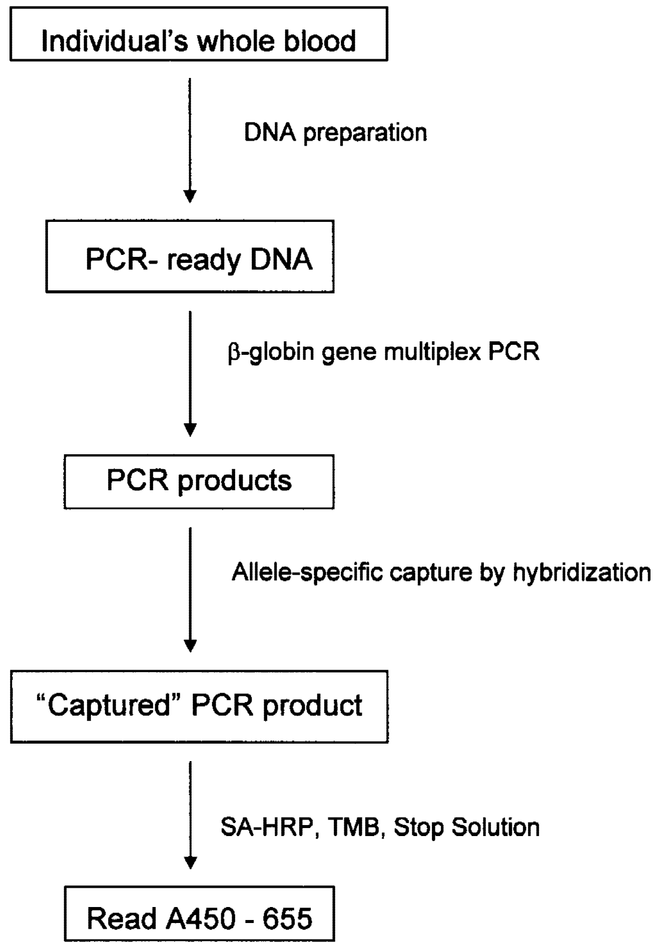

Fig. 1. Overview of the steps involved in the determination of the Mediterranean β-thalassemia genotypes with the BeTha Gene1. SA-HRP is streptavidin-horseradish peroxidate conjugate, and TMB is 3,3′-5,5′ Tetramethylbenzidine.

BeTha Gene 1 kit. A flowchart showing the complete procedure is shown in Figure 1.

Genomic DNA is extracted from an individual's whole blood using a procedure that takes 30 min. After sample preparation, the β-globin gene is amplified in a multiplex PCR reaction containing four 5′ biotinylated primers. The primers for the in vitro amplification of the β-globin gene were chosen to synthesize two segments of the gene: a fragment extending from the 5′ untranslated region to the 5′ end of IVS2; and a 577 bp fragment containing the 3′ end of IVS2 and the third exon (Fig. 2). To detect the PCR products, an aliquot of the PCR reaction is first chemically denatured and then added into 16 wells of a microtiter plate. Each pair of wells contains immobilized ASOs specific for either the normal or mutant allele of a given β-thalassemia mutation site. During a subsequent capture/hybridization step, only perfectly matched PCR products will be captured by an ASO [16]. After washing the wells to remove unbound material, the

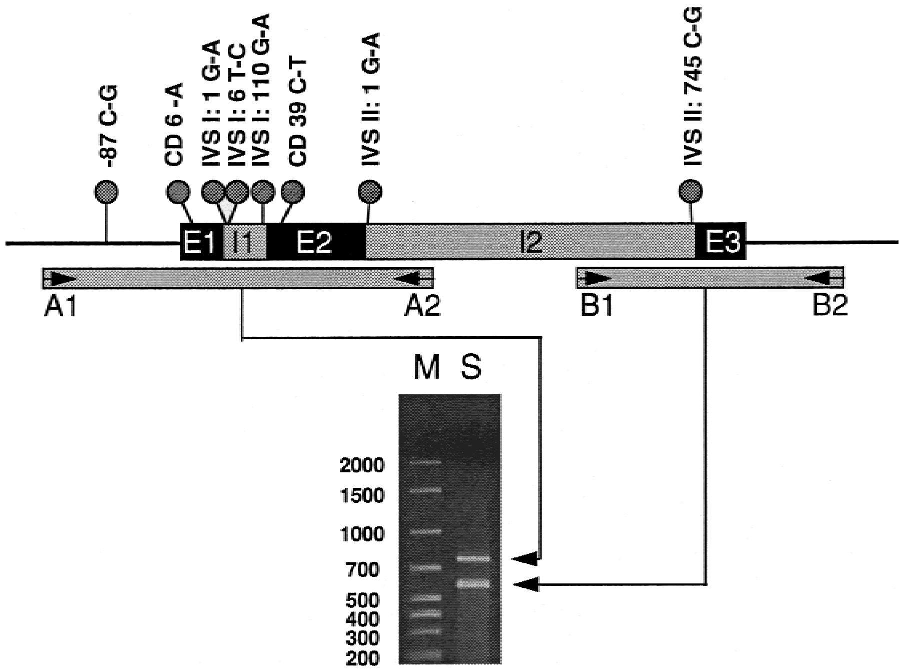

Fig. 2. β-globin gene map. A1-A2 and B1-B2 indicate two pairs of oligonucleotide primers used to amplify β-globin gene fragments of 771 bp and 577 bp, containing the most common mutations in the Mediterranean area. The position of the eight mutations detected by the kit are shown above the gene. The electrophoretic separation of the 771 bp and 577 bp on a ethidium bromide 1.5% agarose gel is shown in the lower part of the figure. M = 50 bp – 2 Kb DNA marker, S = multiplex PCR.

allele-specific captured biotin-labeled PCR products are reacted with SA-HRP conjugate that specifically binds to the biotinylated amplicons. After a final wash to remove unbound enzyme conjugate, the SA-HRP conjugate is detected with the colorimetric substrate TMB. Color development is stopped with acid, converting the blue-colored product to a water soluble yellow product with an absorption maximum at 450 nm.

An example of the four possible outcomes for the analysis of the β-thalassemia mutations is shown in Figure 3a. In the case of a normal individual, a colored signal is generated only in the wells containing the normal ASO probes. An individual that is homozygous for a particular β-thalassemia mutation shows a colored signal with the mutant ASO complementary to that mutation but not with its normal analog, and colored signals with the other seven normal ASOs but not with their mutant analogs. Heterozygous individuals for a single mutation generate a colored signal with both the normal and mutant probes specific for that mutation site and with all other normal ASOs but not with their mutant analogs.

Because so many alleles are found within the Mediterranean population, most individuals with β-thalassemia major carry two different mutations. These compound heterozygous individuals generate a colored signal with two mutant ASOs and with all the normal ASO probes. A distinct interpretation is used for genotypes involving IVS1:1 and IVS1:6, two closely adjacent mutations that fall within the sequence of the same normal ASO probe. In the genotypes IVS1:6 homozygous, IVS1:1 homozygous, and IVS1:6/IVS1:1 compound heterozygous (see Figure 3b), there will be no hybridization to the shared normal ASO probe. For example, the analy-

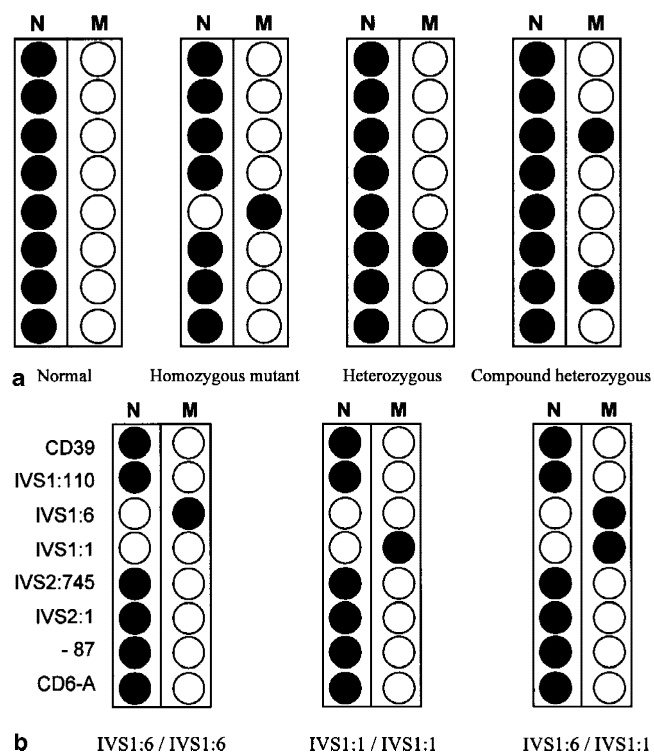

Fig. 3. a: Overview of the four possible outcomes for the analysis of the β-thalassemia mutations. N are the eight normal ASO probes, and M are the eight mutant ASO probes. b: Example of mutations in close proximity: IVS1:6 and IVS1:1.

sis of a homozygous patient for the mutation IVS1:1 will show colored signal with the mutant IVS1:1 ASO probe and absence of colored signal with the normal IVS1:1, normal IVS1:6, and mutant IVS1:6 ASO probes. With a

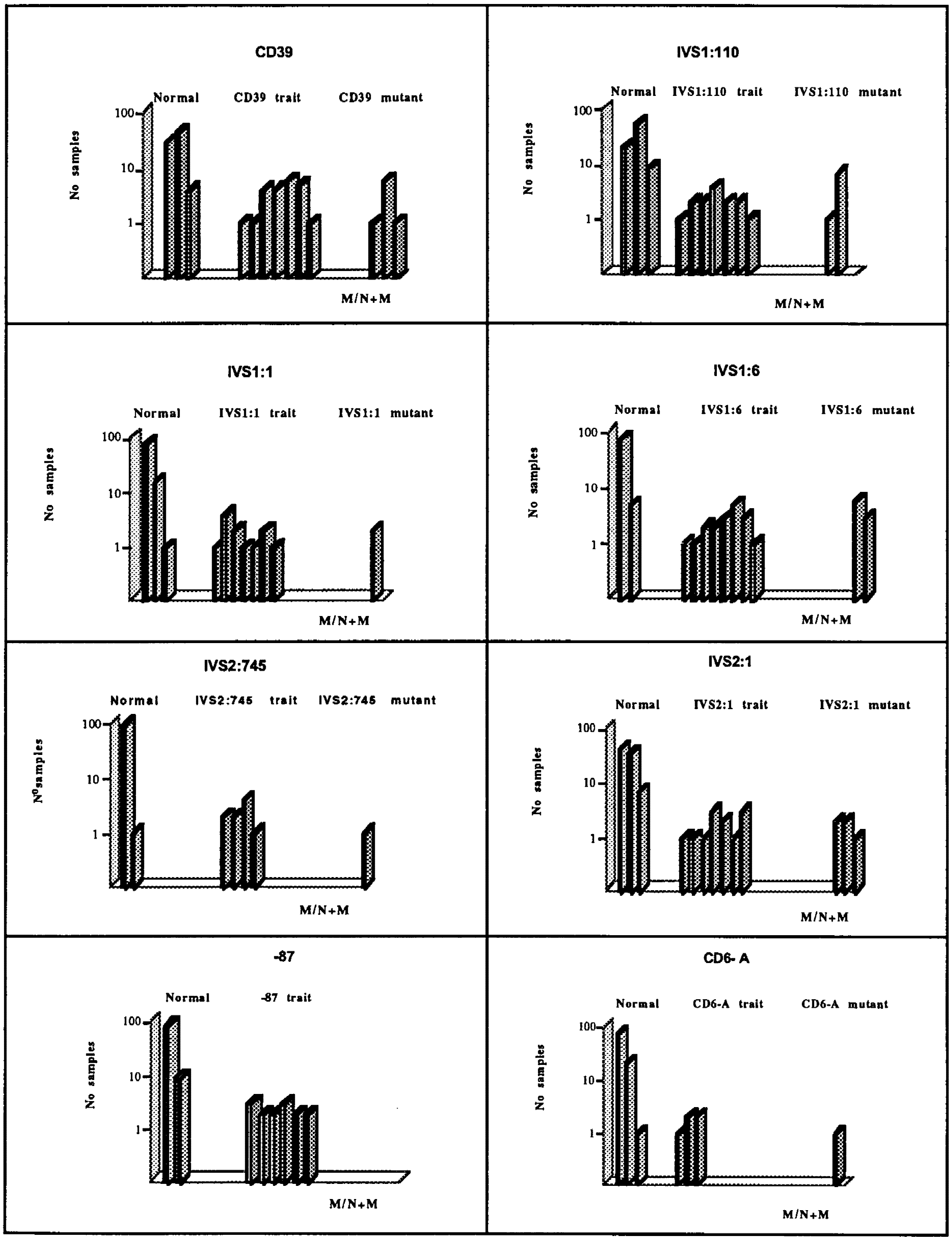

Fig. 4. Data of the method comparison study for 110 DNA samples. BeTha Gene 1kit results were expressed as a ratio of M/N + M where M and N are the A 450/655 for the mutant and normal alleles, respectively. There was perfect correlation between the results generated by the kit and those previously obtained with reference methods.

TABLE II. DNA Titration Data*

	0.5 μl PCR products			1 μl PCR products			3 μl PCR products			5 μl PCR products		
Mutation	N	M	M/N + M	N	M	M/N + M	N	M	M/N + M	N	M/N + M	M
CD39	0.53	0.02	0.04	1.32	0.03	0.02	1.71	0.05	0.03	2.14	0.05	0.02
IVS1:110	1.13	0.03	0.03	1.54	0.05	0.03	2.2	0.09	0.04	2.71	0.05	0.13
IVS1:6	0.98	0.04	0.04	1.35	0.02	0.01	1.75	0.02	0.01	2.21	0.01	0.03
IVS1:1	1.54	0.02	0.01	1.97	0.02	0.01	2.43	0.02	0.01	2.62	0.02	0.04
IVS2:745	0.91	0.02	0.02	1.04	0.02	0.02	0.93	0.03	0.03	0.73	0.06	0.05
IVS2:1	1.01	0.04	0.03	1.48	0.04	0.03	2.21	0.05	0.02	2.45	0.02	0.06
−87	1.54	0.02	0.01	2.33	0.03	0.01	3.1	0.04	0.01	2.91	0.02	0.06
CD6-A	1.21	0.02	0.02	1.35	0.03	0.02	1.61	0.04	0.02	1.51	0.04	0.06

*N = absorbance at 450–655 for the normal ASO probe; M = absorbance at 450–655 for the mutant ASO probe. PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide.

patient homozygous for the mutation IVS1:6, there will be a colored signal with the mutant IVS1:6 ASO probe and absence of colored signal with the normal IVS1:6, normal IVS1:1, and mutant IVS1:1. A IVS1:1/IVS1:6 compound heterozygous will generate a colored signal with both mutant ASO probes and absence of a colored signal with both normal ASO probes.

For the analysis of the mutations CD39, IVS1:110, IVS2:1, IVS2:745, −87, and CD6-A, we calculated the ratio M/[N + M], where M is the A450–655 value for the mutant allele and N is the A450–655 value for the normal allele. Based on results obtained during the developmental phase of the kit, samples were initially classified as normal homozygous when the M/[N + M] ratio was less than 0.11, homozygous mutant when the ratio was greater than 0.89, and heterozygous when the ratio was within the range 0.25–0.75.

A slightly different strategy was used for the analysis of IVS1:1 and IVS1:6. Because of the proximity of the two mutations, the region containing them can be considered as a single locus having three alleles: normal (shared sequence); IVS1:1 mutant; and IVS1:6 mutant. For data analysis, the M/[N + M] ratios for the mutations IVS1:6 and IVS1:1 were calculated using the formulas M 1:6/[N + M1:6 + M1:1] and M1:1/[N + M1:1 + M1:6] respectively, where N is the average of N 1:1 and N 1:6, M1:6 is the A450–655 value for mutant IVS1:6, and M1:1 is the A450–655 value for the mutant IVS1:1.

Discriminative Properties

During kit development, the discriminative properties of all ASO probes were extensively tested for cross-reactivity using the appropriate normal and mutant templates. However, one could argue that the intensities of the signal would be dependent on the amount of the PCR products added into each well possibly resulting in an overlap in the M/N + M range between homozygous and heterozygous samples when detecting large amounts of PCR products. To investigate this hypothesis, a DNA titration experiment was performed. PCR products were generated by amplifying 250 ng of genomic DNA (quantity recommended by the kit's instruction manual) prepared from a normal individual's whole blood. Increasing amounts of PCR products (0.5, 1, 3, and five μl per well) were hybridized to each normal and mutant ASO probe. Results (Table II) show that even when detecting five μl of PCR products, which is an amount 10 times greater than the one recommended by the kit, all the M/N + M ratios fall within the expected ranges indicating no cross-hybridization.

Method Comparison Study

To assess the clinical sensitivity and specificity of the BeTha Gene 1 kit, human genomic DNAs extracted from 110 whole blood samples corresponding to 35 β-thalassemia genotypes were examined (see Table I). The genotypes were previously characterized by the participant collaborators with the methodologies routinely used in their laboratories (reverse dot blot in lab A, DNA sequencing in lab B, and DGGE and dot-blot in lab C). Genotypes not available for this part of the study, IVS1:6/CD6-A, IVS1:1/IVS2:1, and IVS2:1/IVS2:745, were evaluated during the assay validity study for prenatal diagnosis. The genotype −87/−87 was extensively evaluated during kit development (results not shown). Data analysis showed a full concordance between the genotypes determined with the BeTha Gene 1 kit and the results obtained with the reference methods. The correlation between M/[N + M] ratios and genotypes is shown in Table III. The M/[N + M] ratios fell into the three pre-established categories, unequivocally defining the different β-thalassemia genotypes (Fig. 4).

Prenatal Diagnosis Using Chorionic Villus Sampling

Chorionic villus sampling (CVS) is commonly used for fetal DNA sampling [5]. To examine the feasibility of using the kit in the prenatal diagnosis of β-thalassemia mutations, we tested nine DNAs extracted from chorionic villi. One of the samples was eliminated from the study

TABLE III. M/(N + M) Ratios Vs. Genotypes

Genotype	M/N + M (mean ± SD)	Range
CD39 normal (n = 80)	0.05 ± 0.02	0.03–0.11
CD39 heterozygous (n = 22)	0.5 ± 0.09	0.3–0.71
CD39/CD39 (n = 8)	0.94 ± 0.02	0.9–0.97
IVS1:110 normal (n = 85)	0.06 ± 0.02	0.02–0.11
IVS1:110 heterozygous (n = 17)	0.44 ± 0.1	0.25–0.61
IVS1:110/IVS1:110 (n = 8)	0.94 ± 0.01	0.91–0.96
IVS:6 normal (n = 82)	0.03 ± 0.01	0.01–0.06
IVS1:6 heterozygous (n = 18)	0.43 ± 0.07	0.27–0.53
IVS1:6/IVS1:1 (n = 10)	0.96 ± 0.01	0.94–0.98
IVS1:1 normal (n = 96)	0.03 ± 0.01	0.01–0.09
IVS1:1 heterozygous (n = 12)	0.41 ± 0.08	0.31–0.56
IVS1:1/IVS1:1 (n = 2)	0.97 ± 0.03	0.95–0.99
IVS2:745 normal (n = 98)	0.02 ± 0.01	0.01–0.05
IVS2:745 heterozygous (n = 11)	0.5 ± 0.05	0.41–0.56
IVS2:745/IVS2:745 (n = 1)	0.98	
IVS2:1 normal (n = 87)	0.05 ± 0.02	0.02–0.1
IVS2:1 heterozygous (n = 12)	0.42 ± 0.01	0.26–0.55
IVS2:1/IVS2:1 (n = 5)	0.93 ± 0.02	0.91–0.97
−87 normal (n = 103)	0.03 ± 0.01	0.01–0.06
−87 heterozygous (n = 14)	0.46 ± 0.12	0.29–0.68
CD6-A normal (n = 96)	0.04 ± 0.02	0.02–0.09
CD6-A heterozygous (n = 6)	0.36 ± 0.09	0.26–0.52
CD6-A/CD6-A (n = 1)	0.95	

because it could not be amplified by PCR. Agarose gel analysis showed complete degradation of this DNA sample since only DNA fragments of very low molecular weight were observed. The analysis of the other eight samples (genotypes IVS2:745.IVS2:745, IVS1:1/IVS1:1, CD39/IVS1:1, CD39/IVS2:745, IVS1:6/CD6-A, IVS2:745/IVS2:1, IVS1:1/IVS2:1) showed a 100% correlation between the genotypes determined with the BeTha Gene 1 kit and the reference method.

Interfering Genotypes

This study was designed to determine whether various hemoglobinopathy genotypes interfere with kit performance. Results show that sickle cell anemia DNAs did not hybridize with both normal and mutant CD6-A ASO probes and generated a normal hybridization pattern for the other mutations. Genotypes N/−101, N/CD30, N/CD5, N/−88, and N/IVS1:130 generated a normal hybridization pattern. Both genotypes CD39/δβ (Sicilian) and CD39/Hb Lepore generated a CD39 homozygous hybridization pattern.

Assay Validity Using Samples Previously Collected and Stored for Various Periods of Time at Different Temperatures

This study was designed to evaluate assay validity using a normal individual's blood that had been stored in aliquots for various periods of time at different temperatures before DNA preparation. The whole blood aliquots were stored at room temperature up to four days, at 2–8°C up to six days, and at −20°C up to 28 days. All the extracted DNAs were amplified and detected on the same day. Results show that samples that were kept at room temperature for two days, at 2–8°C for six days, and at −20°C for 28 days behaved similarly to the control DNA which was prepared immediately after blood collection.

DISCUSSION

In this paper, we have described the evaluation of the BeTha Gene 1 kit for carrier genotype determination and prenatal diagnosis in DNA samples from Mediterranean patients with β-thalassemia. Our primary goal for conducting this study was to determine whether the kit would produce qualitative results comparable with those obtained by reference methods.

The development of PCR created a revolution within the DNA diagnostic field that facilitated the development of new DNA methods to identify mutations within the β-globin gene. PCR-based methods rely on DNA sequence analysis [17], restriction analysis [18], allele-specific hybridization or amplification [19–21], and polyacrylamide gel electrophoresis [22]. Although these methodologies have been shown to be reliable for the detection of β-thalassemia genotypes, their throughput can be limiting due to their dependence on the use of restriction enzymes, gel electrophoresis, high hybridization temperatures, and plastic bags to perform hybridizations.

We found that the use of the BeTha Gene 1 kit has several advantages over the methodologies previously mentioned. The assay is rapid, with the whole procedure, including DNA preparation, taking about five hr, thus providing the answer in a single working day. In addition, when a confirmation is needed, the colorimetric detection can be repeated within the same day. The enzyme immunoassay (EIA) format selected as the detection system is easy to use, nonradioactive, amenable to automation, and only requires a simple plate reader. In addition, we must emphasize that hybridization is performed at 37°C and washings are conducted at room temperature, which is a great advantage over procedures that use high hybridization and washing temperatures [10] or more than one hybridization temperature [23] to achieve reliable allele-specific discrimination.

As shown in Table III, the results from the method comparison study confirmed the high clinical sensitivity and specificity of the kit. In a study performed on 110 subjects, there was a 100% correlation between the genotypes determined by the kit and the results previously obtained with the reference methods. Forty-two β-thalassemia genotypes were unambiguously determined according to kit specifications (see Table I and prenatal diagnosis study). Kit performance characteristics were also successfully verified on eight DNA samples ex-

tracted from CVS. In analyzing the CVS data, we found no discrepancies between the results determined by the BeTha Gene 1 kit and those obtained with the reference methods.

Whenever a mutation or polymorphism falls within the sequence of a normal ASO, the hybridization of a PCR product could be partially or completely reduced depending on mismatch class and proximity to the β-thalassemia mutation. Therefore, a DNA carrying a mutation or a polymorphic base in close proximity to one of the β-thalassemia mutations within the sequence of an ASO oligonucleotide could behave as an ''interfering genotype.'' For example, DNA samples from individuals with sickle cell anemia (codon 6 is GTG instead of the normal GAG) did not hybridize with both CD6-A normal and mutant ASOs. However, these samples generated a colored signal with all other normal ASO probes but not with their mutant counterparts. A similar situation would occur with a DNA sample from an individual homozygous for Hb C (codon 6 is AAG instead of the normal GAG). Individuals with the genotypes βS/βS, N/βS, βC/βC, βS/βC, and N/βC could be properly identified by hybridizing their DNA samples to mutant βS- or βC-specific probes. As one would expect for mutations located outside the sequence of an ASO, DNA samples from individuals heterozygous for the mutations −101, CD30, and IVS1:130 generated a normal hybridization pattern. Although the mutations −88 and CD5 fall within the sequence of the −87 and CD6-A ASO probes respectively, DNAs from N/88 and N/CD5 individuals generated normal hybridization patterns. Obviously, the amplification of the normal β-globin gene in the unaffected chromosome of these individuals masks the interfering effect that the −88 and the CD5 mutations would have if they had been present in the homozygous state.

Deletions or rearrangements of different sizes that totally or partially remove the β-globin gene may cause a failure in the synthesis of either one or both PCR products. Hb Lepore Boston, an abnormal Hb thought to be produced by an unequal crossover between the δ- and the β-globin genes, is another example of an interfering genotype. In fact, the unequal crossover generates a deletion approximately 950 bp long that includes the entire intervening sequence [IVS:2] [24] thereby preventing the synthesis of the 771 bp PCR fragment. Individuals heterozygous for Hb Lepore and any one of the seven β-thalassemia mutations included within the 771 bp fragment are hemizygous for this DNA fragment; that is, only the chromosome carrying the β-thalassemia mutation is amplified by PCR. PCR products from these individuals will hybridize to the mutant ASO probe but not to the normal ASO probe for that particular mutation site. As an example of a β-thalassemia/Hb Lepore Boston compound heterozygote we studied DNA from a CD39/Hb Lepore heterozygous subject. For this particular genotype, the BeTha Gene 1 kit generated a CD39/CD39 homozygous mutant hybridization pattern. In contrast, because the synthesis of the 577 bp PCR fragment is not affected by Hb Lepore, PCR amplification products from a heterozygous individual for Hb Lepore/IVS2:745 will hybridize to both normal and mutant IVS2:745 ASO probes.

Sicilian δβ-thalassemia, which is characterized by a deletion starting from the δ-globin intron and extending several kilobases 3′ to the β-globin gene [25], is another example of an interfering genotype. In this particular case the synthesis of both PCR products is prevented. Because the chromosome segment containing the δβ deletion cannot be amplified, a sample from an individual that is compound heterozygous for CD39/Sicilian δβ-thalassemia was genotyped by the BeTha Gene 1 kit as CD39 homozygous mutant.

It is noteworthy that the kit is designed to determine the presence of known mutations, more specifically the eight most common Mediterranean β-thalassemia mutations. Mutations outside the sequence of the ASO probes or outside the sequence amplified by the multiplex PCR, DNA deletions or rearrangements, and unknown mutations are not detected by this assay. Because the method is limited to eight mutations, patients carrying rare or unknown mutations cannot be diagnosed with this kit. For these patients, characterization of β-thalassemia could be performed by using other methods.

CONCLUSION

In summary, the data from this study show that this sensitive and specific method provides satisfactory performance characteristics for the correct identification and prenatal diagnosis of individuals carrying the eight most common Mediterranean β-thalassemia mutations. This nonradioactive assay is safe, rapid, and will yield results in 5–6 hr.

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